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ORIGINAL ARTICLE

Transmission of cotton seed and boll rotting bacteria by the southern green stink bug (*Nezara viridula* L.)

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Abstract

Aims: To determine the ability of the southern green stink bug (SGSB) (*Nezara viridula* L.) to transmit *Pantoea agglomerans* into cotton (*Gossypium hirsutum*) bolls.

Methods and Results: An SGSB laboratory colony was kept on fresh green beans. A *P. agglomerans* variant resistant to rifampicin (Rif) (strain Sc 1-R) was used as the opportunistic cotton pathogen. Adult insects were individually provided green beans that were sterilized and then soaked in either sterile water or in a suspension of strain Sc 1-R. Insects were individually caged with an unopened greenhouse-grown cotton boll. After 2 days, live SGSB were collected, surfaced sterilized, ground, serially diluted, and then plated on nonselective media and media amended with Rif. Exterior and interior evidence of feeding on bolls was recorded 2 weeks after exposure to insects. Seed and lint tissue were harvested, ground, serially diluted, and then plated on media with and without Rif. Bacteria were recovered on nonselective media from all insects, and from seed and lint with signs of insect feeding at concentrations ranging from 10^2 to 10^9 CFU g^{-1} tissue. The Sc 1-R strain was isolated only from insects exposed to the marked strain and from seed and lint of respective bolls showing signs of insect feeding. Evidence of insect feeding on the exterior wall of the carpel was not always apparent (47%), whereas feeding was always observed (100%) on the interior wall in association with bacterial infections of seed and lint.

Conclusions: *Nezara viridula* readily ingested the opportunistic *P. agglomerans* strain Sc 1-R and transmitted it into unopened cotton bolls. Infections by the transmitted Sc 1-R strain caused rotting of the entire locule that masked internal carpel wounds incurred by insect feeding. Bacteria were recovered from penetration points by insects not exposed to the pathogen, but locule damage was limited to the area surrounding the feeding site (*c.* 3 mm).

Significance and Impact of the Study: This is the first study that demonstrates the ability of SGSB to acquire and transmit plant pathogenic bacteria into cotton bolls.

Introduction

Seed and boll rot caused by nontraditional cotton (*Gossypium hirsutum*) pathogens was first reported in 1999 and has become an important factor causing significant yield losses in the south-eastern US Cotton Belt, particularly in South Carolina (Hudson 2000; Hollis 2001). The exterior walls of carpels of unopened diseased bolls are nonsymptomatic and hence complicate early detection of affected fields. Cross-sections of diseased fruit reveal necrotic seed and discoloured fibre tissue (Edmisten 1999). Both diseased and healthy locule(s) may occur in a single boll (typically 3–5 locules/boll). Partially diseased bolls have matted discoloured lint with deteriorated seed in infected locule(s) and typical fluffy white fibre with normal seed in noninfected locule(s)

(Mauney and Stewart 2003; Mauney *et al.* 2004). Yield losses result from premature senescence of partially or totally infected bolls and from inefficient harvesting of bolls with matted lint by mechanical pickers (Hudson 2000).

The national boll weevil eradication programme and the introduction of transgenic Bt (*Bacillus thuringiensis*) toxin-producing cotton have decreased yield losses caused by boll weevils (*Anthonomus grandis* B.) and the Heliothine complex (bollworm, *Helicoverpa zea* B./tobacco budworm *Heliothis virescens* F.) (Greene *et al.* 1999; Bundy and McPherson 2000; Jackson *et al.* 2004), respectively. The considerable reduction of cotton damage by these traditional pests has resulted in decreased pesticide applications and decreased use of broad-spectrum insecticides (Barbour *et al.* 1990; Bundy and McPherson 2000) and, as a consequence, a notable increase in yield losses to formerly 'minor' pests such as the piercing/sucking insects. As contributors to cotton yield losses, stink bugs (Heteroptera: Pentatomidae) ranked third following the Heliothine complex and lygus bugs (Heteroptera: Miridae) during 2005 (Williams 2005).

Stink bugs feed by inserting their interlocking mandibular and maxillary stylets into a food source to ingest nutrients (Panizzi *et al.* 2000). Digestive enzymes are injected via the salivary canal and liquefy host tissue as stylets cut through the tissue. Stink bugs became important pests of cotton because of their piercing/sucking mode of feeding, lack of specific control measures, and other factors described above.

We demonstrated the role of *Pantoea agglomerans* in opportunistic bacterial seed and boll rot of cotton by fulfilling Koch's postulates (Medrano and Bell 2007). In the greenhouse study, a syringe and needle were used to inject a suspension of *P. agglomerans* rifampicin (Rif)-resistant mutant (Sc 1-R) into healthy grown bolls. Harvesting inoculated bolls 2 weeks after exposure to the bacterium revealed infection symptoms analogous to those observed in diseased field-grown South Carolina fruit from which the *P. agglomerans* parental-type was isolated. Inoculations that did not puncture the boll did not result in disease development. These observations indicated that a wound similar to that caused by stink bugs was needed for the disease to occur. Therefore, the southern green stink bug (SGSB) (*Nezara viridula* L.) was examined as a possible vector of seed and boll rotting bacteria.

Materials and methods

Bacterial exposure to insects

Feral adults of *N. viridula* were collected from commercial soybean [*Glycine max* (L.) Merr.] fields to establish a laboratory colony. Colony adults, eggs and immature stages were held at 28°C and a 14 : 10 (L : D) photoperiod. All colony adults and immature stages were fed fresh green bean (*Phaseolus vulgaris* L.) pods thoroughly rinsed in a 10% sodium bicarbonate solution before offered to the bugs, providing a presumably disease-free food source. Beans were replaced at 2-day intervals. *Pantoea agglomerans* strain Sc 1-R, a demonstrated cotton pathogen marked with Rif resistance (Medrano and Bell 2007), was used in insect transmission studies and routinely maintained on Luria–Bertani agar (LBA) (Difco Laboratories, Detroit, MI, USA) amended with Rif (100 μg ml^{-1}) and grown at 28°C.

Suspensions of strain Sc 1-R were prepared in sterile water from 18-h cultures and adjusted spectrophotometrically ($A_{600} = 1{\cdot}0$). A millilitre of the concentrated bacterial suspension was added to 49 ml sterile water and used as the inoculum. In preparation for bacterial exposure experiments, fresh green beans were sterilized (121°C, 20 min, 1 kg cm^2) and then cross-sectioned into pieces (*c.* 3 cm) using a sterile scalpel (bean ends were discarded). To test for sterility, samples from each batch processed were triturated using a sterile mortar and pestle with 1 ml of water, plated on LBA and potato dextrose agar (PDA) (Difco), and incubated for no longer than 2 weeks at 28°C. Green bean sections provided to insects were soaked for 2 min in either sterile water or the Sc 1-R suspension, blotted dry using sterile paper towels, and placed individually in a sterile, disposable Petri dish (100 × 15 mm). A single insect of determined sex was placed in a Petri dish containing a treated green bean section and maintained in the environmental chamber using the parameters listed above. Insects were exposed to treated green beans for 7 days. Replacement beans were added to a fresh Petri dish, and insects were transferred using sterile forceps (i.e. ethanol dipped and then flamed). Green beans soaked in the bacterial suspension were provided to insects for 2, 3, 6, or 7 days. Following the designated bacterial exposure period, sterile beans were used to complete the 7-day trial.

Treated insects were individually transferred into a 14-ml round bottom Falcon tube (Becton Dickinson, Franklin Lakes, NJ, USA) that contained 10 ml of 70% ethanol for surface sterilization. After 8 min of gentle inversion of the tubes, the insects were rinsed twice for 1·5 min in 25 ml sterile water and then transferred into a 1·1 ml microtube (SPEX SamplePrep, Metuchen, NJ, USA) that contained 0·5 ml PO_4 buffer (0·1 mol l^{-1}, pH 7·1) and a sterile 4 mm stainless steel ball (SPEX SamplePrep). A second sterile 4 mm stainless steel ball was added and the capped 1·1-ml microtubes from strips of eight were placed in a rack of 96 tubes for grinding. To test for the presence of micro-organisms in the wash

water, 100 μl aliquots were directly plated on LBA and PDA then incubated for 1 week at 28°C. The insects were ground using a 2000 Geno/Grinder (SPEX SamplePrep) for 5 min at 1500 strokes min^{-1} then dilution (PO_4 buffer, pH 7·1) plated on both LBA and LBA amended with Rif. After 2 days of incubation at 28°C, bacterial colonies were enumerated and recorded as CFU g^{-1} insect tissue.

Insect infestation on cotton bolls

Beginning in August 2005, cotton plants (cv. Coker 312) were propagated from seed in a greenhouse using the methods and parameters described in Medrano and Bell (2007). Plants were protected from feral insect injury with weekly spraying of Avid 0·15EC Miticide/Insecticide (Syngenta Crop Protection, Inc., Greensboro, NC, USA) and Provado 1·6 Flowable Insecticide (Bayer CropScience LP, Research Triangle Park, NC, USA) following the manufacturer-recommended concentration rate until 10 days before conducting stink bug infestation experiments. All bolls used in transmission studies were at a maturity of 2 weeks post-anthesis with a maximum of three caged/plant. Before and after caging a boll with a stink bug, representative fruit samples were tested for the presence of Rif-resistant bacteria on the carpel by imprinting bolls on LBA amended with Rif. Bacterial growth was recorded following a 2-day incubation period at 28°C.

Initial studies of insect exposure to bacteria revealed that strain Sc 1-R could be detected in stink bugs after providing pathogen-contaminated beans for all the time periods tested. Therefore, insects exposed to Sc 1-R-treated beans were caged over cotton bolls for 2 or 6 days. Negative controls were included in all experiments and consisted of insects that had been fed for 2 or 6 days sterile beans soaked in water. Insects were held without food for 24 h before caging with cotton bolls to increase the probability of feeding.

Insect cages consisted of a 6-oz (170 g) foam cup (WinCup, Phoenix, AZ, USA), sections of knee-high nylon hose, and twist ties. A cup, hollowed at the bottom end, was placed (narrow end facing plant) over a boll and enclosed with the nylon hose (hollow section) that was then sealed with twist ties both at the petiole and narrow end of the cup. A single stink bug was inserted into each cage and held for a period of either 2 or 5 days. Following the exposure times, live insects were tested for the presence of the Sc 1-R strain using the methods described above and data were recorded as CFU g^{-1} insect tissue.

Cotton boll injury

Cages were re-sealed after removal of the stink bugs and the cotton bolls were harvested 2 weeks after exposure to the bugs. Bolls were individually surface sterilized for 10 min in a 0·5% sodium hypochlorite solution then rinsed for 2 min in sterile water three times. Evidence of insect feeding on the inner and outer carpel, and symptoms of infection on lint and seed tissue were recorded after excising carpel walls with a sterile scalpel. Lint and seed (*c.* 0·5 g) from locules with signs of insect feeding were diced and transferred into a 1·1 ml microtube that contained 0·5 ml PO_4 buffer and a sterile 4 mm stainless steel ball. A second 4 mm stainless steel ball was added, and the tissue was ground using a 2000 Geno/Grinder (SPEX SamplePrep) for 10 min at 1500 stroke min^{-1} then dilution (PO_4 buffer, pH 7·1) plated on both LBA and LBA amended with Rif. Seed and lint tissue from bolls caged without a stink bug were processed as negative controls. After 2 days of incubation at 28°C, bacterial colonies were enumerated and recorded as CFU g^{-1} plant tissue.

Characterization of bacterial isolates

Remote colonies recovered from insects or plant tissues were purified and stored at −80°C in a 40% glycerol solution diluted with 1% LB. A 16S ribosomal PCR-amplified product was cloned and sequenced for representative isolates using the protocols described in Medrano and Bell (2007). Derived 16S rDNA gene sequence data from both strands were edited and assembled using SEQUENCHER 4·2 (Gene Codes Corp., Ann Arbor, MI, USA) then compared with sequences in the GenBank database using the BLAST program offered by the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST/).

Results

Bacterial exposure to insects

Bacteria were isolated from all insects with a concentration range of 10^5–10^9 CFU g^{-1} insect tissue (Table 1). Rifampicin-resistant bacteria were not recovered from insects that were used as negative controls (i.e. not exposed to Sc 1-R). Micro-organisms were not detected in the sterilized green beans or in the sterile water used to rinse the surfaced-sterilized beans.

The highest concentrations (10^5 CFU g^{-1} tissue) of the Rif-resistant Sc 1-R bacteria were recovered from insects fed contaminated green beans for 6 or 7 days. There was no effect of Sc 1-R on insect vigour. The Sc 1-R bacteria were detected in an average of 75% of insects exposed to contaminated beans for 2 or 3 days and then fed sterile beans without bacteria for 5 days thereafter (Table 1). There were no statistically significant differences in Sc 1-R

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Table 1 Bacterial concentration (CFU g^{-1}) ranges recovered from surface-sterilized *Nezara viridula* provided a sterile food source for 7 days or food inoculated with a bacterial suspension (i.e. strain Sc 1-R) and then offered to the stink bugs for a maximum of 7 days

	LBA*		LBA Rif†	
Days exposed to Sc 1-R‡	Lowest bacterial§ concentration	Highest bacterial concentration	Lowest bacterial concentration	Highest bacterial concentration
0 days				
Males ($n = 8$)	10^6	10^9	$<10^1$	$<10^1$
Females ($n = 8$)	10^6	10^9	$<10^1$	$<10^1$
2 days				
Males ($n = 8$)	10^6	10^9	$<10^1$	10^3
Females ($n = 8$)	10^5	10^8	$<10^1$	10^3
3 days				
Males ($n = 8$)	10^6	10^9	$<10^1$	10^3
Females ($n = 8$)	10^6	10^8	$<10^1$	10^3
6 days				
Males ($n = 8$)	10^6	10^9	10^3	10^5
Females ($n = 8$)	10^5	10^9	10^2	10^4
7 days				
Males ($n = 8$)	10^6	10^9	10^3	10^5
Females ($n = 8$)	10^6	10^9	10^3	10^5

*Luria–Bertani agar.
†LBA amended with rifampicin (100 μg ml^{-1}).
‡Exposure to Sc 1-R involved offering the insects green beans that had been sterilized and then soaked in a bacterial suspension; insects were offered treated green beans for 7 days and sterile beans were used to complete exposure periods that were <7 days.
§Bacterial concentrations were expressed as CFU g^{-1} insect tissue.

concentrations found among insects exposed to contaminated beans for 2, 3, 6 or 7 days (F was only significant at $P < 0{\cdot}01$). Furthermore, concentrations of Sc 1-R in male and female stink bugs were not significantly different (F was only significant at $P < 0{\cdot}05$).

Insect infestation on cotton bolls

Sixty-eight of 100 (60 treated and 40 control insects) bugs survived the caged period of 2 or 5 days. Survivors were processed and plated on both selective and nonselective media. The highest bacterial concentrations measured on media without Rif was 10^9 g^{-1} tissue from control males and 10^8 g^{-1} tissue from females. Conversely, no bacteria were recovered from control insects on media amended with Rif (Table 2).

Bugs ($n = 26$) exposed to the Sc 1-R strain for 2 days were collected from cage experiments after 2 or 5 days (Table 2). In accordance with preliminary experiments (Table 1), bacteria were recovered from all insects on LBA with a range of 10^5–10^9 CFU g^{-1} tissue, and Sc 1-R was detected on both LBA and LBA amended with the antibiotic (Table 2). Furthermore, strain Sc 1-R was readily recovered from insects at concentrations of 10^2–10^3 g^{-1} tissue 7 days after their last exposure to the bacteria-contaminated beans for 2 days. After exposure, insects were kept for 5 days with sterile green beans in the laboratory and 2 days over cotton bolls in the greenhouse. Stink bugs exposed to Sc 1-R-contaminated beans for 6 days compared with 2 days before caging with cotton bolls for 2 days contained 100-fold increases of the marked strain (i.e. 10^5 CFU g^{-1} compared with 10^3 CFU g^{-1} tissue).

A total of 18 insects (9 males and 9 females) survived the 2- or 5-day caging over cotton bolls following a 6-day exposure to Sc 1-R-contaminated sterile beans (Table 2). The highest levels of bacteria detected in insects exposed to Sc 1-R-soaked beans before being caged for 2 or 5 days with bolls were 10^6 and 10^9 g^{-1} tissue on nonselective media, respectively. Strain Sc 1-R was recovered from stink bugs caged 2 or 5 days at concentrations of 10^5 and 10^3 CFU g^{-1} tissue, respectively.

Cotton boll injury

Bacteria were not recovered from carpel imprints on LBA amended with Rif using representative bolls before or after caging with any insects tested. From the bolls analysed ($n = 100$) for signs of insect feeding, 42 had puncture wounds on the carpel wall and/or damaged lint tissue. Only 20 bolls showed detectable puncture lesions on the exterior carpel wall, whereas all 42 showed damage of the inner carpel wall. Of the bolls with lesions on the outer carpel, 95% had 1–2 punctures per boll in the

Table 2 Bacterial concentration (CFU g^{-1} tissue) ranges recovered from *Nezara viridula* exposed to strain Sc 1-R via a food source (excluding controls), and then individually caged with an unopened greenhouse grown cotton boll

	LBA*		LBA Rif†	
Days insect caged with a cotton boll‡	Lowest bacterial§ concentration	Highest bacterial concentration	Lowest bacterial concentration	Highest bacterial concentration
Control insects¶ ($n = 24$)				
Caged 2 days	10^6	10^9	$<10^1$	$<10^1$
Caged 5 days	10^6	10^9	$<10^1$	$<10^1$
Treatment I** ($n = 26$)				
Caged 2 days	10^6	10^9	$<10^1$	10^3
Caged 5 days	10^5	10^8	$<10^1$	10^2
Treatment II†† ($n = 18$)				
Caged 2 days	10^6	10^9	10^2	10^5
Caged 5 days	10^6	10^8	10^2	10^3

*Luria–Bertani agar.

†LBA amended with rifampicin (100 μg ml^{-1}).

‡After being subjected to a specific dietary treatment (described below), insects were individually caged with a cotton boll.

§Bacterial concentrations were expressed as CFU g^{-1} insect tissue.

¶Prior to the cage experiments, the control insect dietary treatment included green beans that had been sterilized before being offered to the insects for 6 days (replenished every 2 days) followed by holding the insects without food for 24 h.

**Prior to the cage experiments, the insect dietary treatment I included green beans that had been sterilized and then soaked in a bacterial suspension before offered to the insects for 2 days; the bacteria-treated beans were replenished with sterile beans for 4 days (replenished every 2 days), and finally the insects were held for 24 h without food.

††Prior to the cage experiments, the insect dietary treatment II included green beans sterilized and then soaked in a bacterial suspension before offered to the insects for 6 days (replenished every 2 days) followed by holding the insects without food for 24 h.

carpel wall. The remainder had three or more punctures with a maximum of nine wounds. Numbers of inner carpel punctures ranged from 1 to 28 per boll with 95% of bolls having 1–5 lesions and 5% having greater than five lesions. Individual bolls had both damaged and apparently unaffected locule(s).

Eighty per cent of the inner carpel lesions inflicted by control insects had no juxtaposed locule tissue damage based on discoloured lint and seed (Fig. 1). Of the characteristic blisters from punctures through the inner capsule by control insects, 60% were covered by a yellow callus or wart-like formation. Infiltration of the bolls by insects exposed to strain Sc 1-R resulted in necrosis of 85–95% of the inner carpel epidermal tissue (Fig. 1). Necrotic tissue and black, bulbous calluses were distributed throughout the inner capsule wall and masked characteristic blisters. Damage of the inner carpel consistently resulted in discoloration of the entire locule tissue. All embryos in seeds infected with Sc 1-R were deteriorated, whereas embryos in seeds punctured by control insects (i.e. not exposed to Sc 1-R) were conspicuously less affected (Fig. 2).

Bacteria were not detected when control lint or seed tissue were plated from insect-free bolls on nonselective media (Table 3). Conversely, bacteria were recovered on LBA from all bolls at the site with evidence of insect feeding and lint and seed discoloration at concentrations ranging from 10^2 to 10^8 CFU g^{-1} tissue (Table 3). Damage to locule tissue by control insects only occurred in the area immediately surrounding the puncture wound (Fig. 1). Strain Sc 1-R was both exclusively and persistently isolated from locule tissue penetrated by insects exposed to the pathogen for 2 or 5 days and then caged over cotton bolls for 2 or 5 days. Detection of strain Sc 1-R always coincided with rotting of the entire locule, whereas unaffected tissue in adjacent locule(s) showed no apparent evidence of insect probing or bacterial infection.

Characterization of bacterial isolates

A 1·5-kb portion of the 16S ribosomal RNA gene was cloned and sequenced for 20 bacterial isolates with different colony morphologies. Eight of the representatives were isolated directly from the insects and the rest from seed and lint tissue with signs of feeding by control insects. Based on the BLAST analysis, the sequence data acquired for bacteria cultured from locule tissue injured by insects and/or insects had identities of 97% (or greater) with *Serratia*, *Staphylococcus*, *Pseudomonas*, *Pantoea*, or *Enterobacter* species. The same species plus *Bacillus* spp. were isolated from symptomatic locules.

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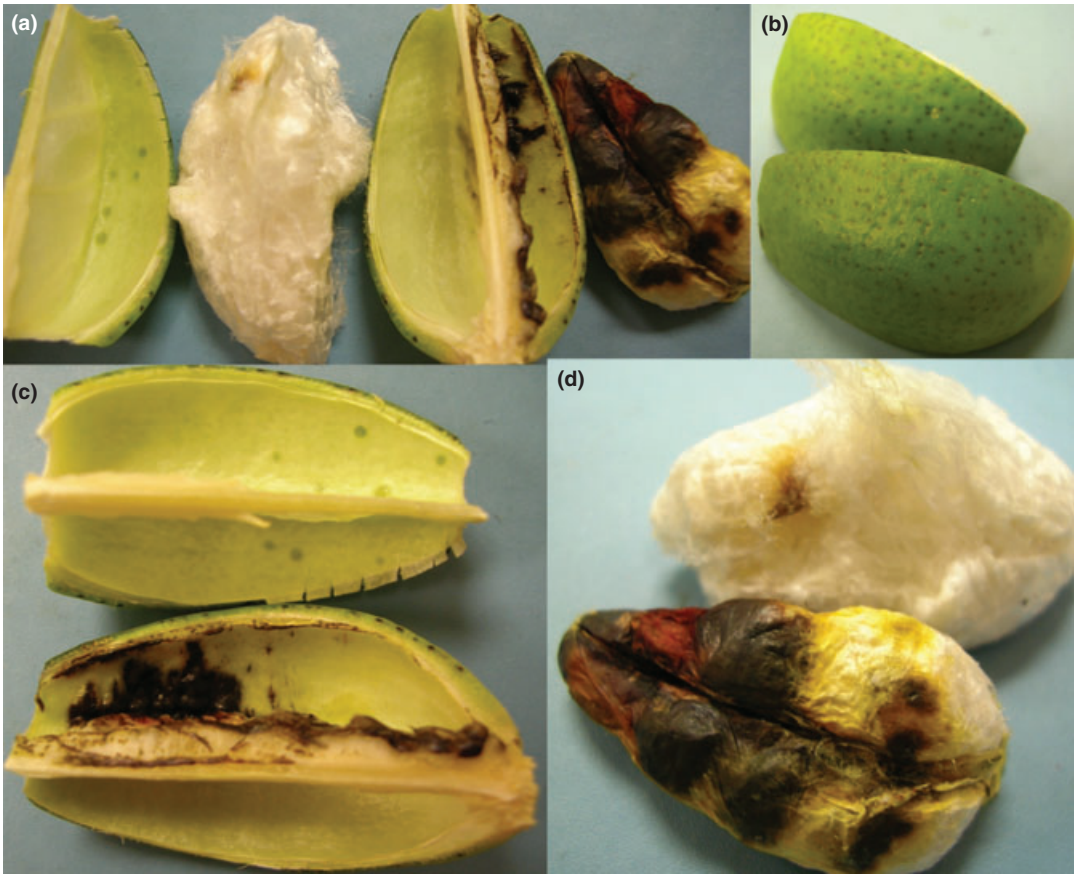

Figure 1 External and internal effects of feeding on greenhouse grown cotton bolls by *Nezara viridula* not harbouring strain Sc 1-R and harbouring the bacterial pathogenic strain. Bolls were 2 weeks post-anthesis when caged with stink bugs for 2 days; symptoms were photographed 2 weeks after exposure to insect. (a) The boll on the left is an example of inner carpel and locule damage associated with control stink bug feeding and the boll on the right illustrates effects of feeding by a stink bug that was exposed to the Sc 1-R bacteria via a contaminated food source. (b) Effects of probing on the external carpel by a control insect (top section) and a stink bug that transmitted strain Sc 1-R (bottom section). (c) Effects of probing on the internal carpel (inner view of same samples in photograph b) by a control insect (top section) and a stink bug that transmitted strain Sc 1-R (bottom section). (d) Effects of feeding on locules by a control insect (top section) and a stink bug that transmitted strain Sc 1-R (bottom section).

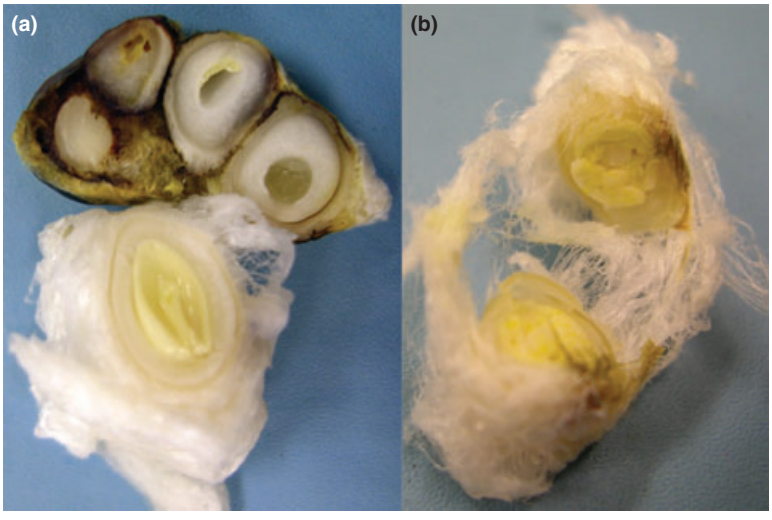

Figure 2 Differences in seed development from greenhouse grown cotton bolls caged with no insect, with *Nezara viridula* not harbouring a bacterial pathogen, or with *N. viridula* harbouring strain Sc 1-R. Bolls were 2 weeks post-anthesis when caged with insects, and photographed 2 weeks after the experiments. (a) A seed from an Sc 1-R-infected locule with evidence of feeding by a stink bug harbouring the plant pathogen (top) and seed from a boll caged without insect (bottom). (b) Example of seeds from a locule with signs of feeding by a control insect (i.e. not exposed to strain Sc 1-R). Note: embryoes are deteriorated within seed (panel a, top) only when infected by Sc 1-R.

Table 3 Bacterial concentration (CFU g^{-1}) ranges recovered from control locules harvested from cotton bolls not exposed to insects and from locules of bolls with evidence of *Nezara viridula* feeding

	LBA*		LBA Rif†	
Days insect caged with a boll‡	Lowest bacterial§ concentration	Highest bacterial concentration	Lowest bacterial concentration	Highest bacterial concentration
Control bolls ($n = 6$)	$<10^1$	$<10^1$	$<10^1$	$<10^1$
2 days ($n = 26$)				
Insect T¶	10^2	10^6	$<10^1$	$<10^1$
Insect TI**	10^3	10^8	10^4	10^8
Insect TII††	10^6	10^8	10^6	$<10^8$
5 days ($n = 16$)				
Insect (control)	10^2	10^7	$<10^1$	$<10^1$
Insect TI	10^4	10^8	10^4	10^8
Insect TII	10^6	10^8	10^6	10^8

*Luria–Bertani agar.

†LBA amended with rifampicin (100 μg ml^{-1}).

‡Bolls were at a maturity of 2 weeks post-anthesis and harvested 2 weeks after insects were removed from the cage.

§Bacterial concentrations were expressed as CFU g^{-1} insect tissue.

¶Prior to the cage experiments, the control insect dietary treatment (T) included green beans sterilized before offered to the insects for 6 days (replenished every 2 days) followed by holding the insects without food for 24 h.

**Prior to the cage experiments, the insect dietary treatment I (TI) included green beans sterilized and then soaked in a bacterial suspension before being offered to the insects for 2 days; the bacteria-treated beans were replenished with sterile beans for 4 days (replenished every 2 days), and finally the insects were held for 24 h without food.

††Prior to the cage experiments, the insect dietary treatment II (TII) included green beans sterilized and then soaked in a bacterial suspension before being offered to the insects for 6 days (replenished every 2 days) followed by holding the insects without food for 24 h.

Discussion

The SGSB colonies were raised in a nonsterile environmental chamber and on nonsterile, washed, and apparently healthy green beans. Therefore, detection of bacteria from control insects not exposed to Sc 1-R and that had been surface sterilized was expected (Table 1). In a study of SGSB digestive tract bacterial flora, Hirose *et al.* (2006) reported levels of bacteria from a 3-year-old laboratory-reared colony that ranged from 10^3 to 10^8 CFU ml^{-1}; thus, although data were expressed differently, CFU numbers are generally comparable with our results. Furthermore, the Hirose *et al.* (2006) study states that bacteria recovered from the surgically removed stink bug midgut were categorized as *Klebsiella pneumoniae*, *Enterococcus faecalis* and a putative *Pantoea* sp. Differences between the types of bacteria reported in the current study can be attributed to several variables including insect processing methods used (surgical midgut removal *vs* grinding of whole insect), diet (green beans and sunflower seeds *vs* grean beans only), and time period insect colonies had been maintained in captivity.

Providing insects green beans first immersed in a solution contaminated with strain Sc 1-R was an effective method for infesting insects with the cotton pathogen. Insect vigour did not differ between control insects and insects that acquired the Sc 1-R strain. Males and females acquired the marked strain at similar frequencies. Thus, acquisition and harbouring of the cotton pathogen did not adversely affect the health of the insects subjected to Sc 1-R exposure, regardless of sex. Strain Sc 1-R was detectable from stink bugs maintained for a total 10 days (5 days in the laboratory and then 5 days in the greenhouse) after exposure to the contaminated food source for only 2 days suggesting that the mutant was capable of at least transiently colonizing the insects. If colonization occurs in the rostrum (i.e. the feeding apparatus), stink bugs theoretically have the capacity to transmit pathogens to multiple bolls in the field.

Boll feeding by control insects resulted in some seed and lint damage as estimated (Fig. 1). In accordance with previous reports (Greene *et al.* 2001; Willrich *et al.* 2004), characteristic callus formation and blistering of internal carpel tissue from insect punctures was not always accompanied by visible damage to the outer boll epidermal layer. The number of puncture wounds inflicted by male and female insects was not significantly different; both sexes consistently introduced bacteria into locule tissue during feeding. Interestingly, concentrations of bacteria recovered from seed and lint tissue with evidence of

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feeding by control insects reached concentrations as great as 10^6 CFU g^{-1} locule tissue in spite of only a local discoloration of the tissue about the wound site (Fig. 2). Bacteria were not detected in a lock with apparently undamaged lint and seed tissue that surrounded the feeding site of control insects (data not shown) indicating a lack of movement of the micro-organisms outside the damaged region. The occurrence of *Bacillus* spp. in locule tissue damaged by insect feeding but not in the insects may be attributed to stink bugs acquiring plant surface microflora from probing during the cage experiments.

Strain Sc 1-R was not recovered on selective media from imprints of bolls regardless of whether the fruit had been caged for 2 or 5 days with insects previously exposed to the Sc 1-R pathogen. This indicated that entry of the mutant into bolls occurred via feeding and not incidentally due to a contaminated exterior boll carpel. As with the control insects, outer carpel damage was not a reliable indicator of boll puncturing by insects first provided Sc 1-R-contaminated green beans (Fig. 1). Damage to bolls following feeding by insects that vectored the bacterial pathogen included the entire punctured locule, indicating that infection spread from the point of inoculation. Furthermore, the resulting necrosis of the locule and inner carpel masked the blistering from insect punctures and thus deviated from evidence typically associated with SGSB injury. Notably, the disease symptoms depicted in Figs 1 and 2 are comparable with bacterial infection symptoms reported in Medrano and Bell (2007) following mechanical inoculations using the same Sc 1-R strain. An additional consistency with Medrano and Bell (2007) is containment of the disease to punctured locules (i.e. locules on the same boll devoid of piercing wounds possessed typical seed and lint tissue).

The *P. agglomerans* Rif-resistant derivative (i.e. Sc 1-R) was routinely isolated from surface-sterilized SGSB that had been provided an Sc 1-R-contaminated food source for 2 or 6 days. Bacteria were consistently detected from all locule tissues with signs of insect feeding on nonselective media. Thus, feeding was found to be a viable mechanism for bacterial acquisition and transmission between food sources. Therefore, stink bugs are potential vectors of bacterial plant pathogens in field infections. The inner diameter of the insect's feeding channel in the rostrum is of ample width (0·01 mm) for bacterial cells to move through the piercing apparatus (Ragsdale *et al.* 1979).

Ashworth *et al.* (1969) associated brown stink bug (*Euschistus servus* S.) feeding with transmission of *Erwinia* sp. (Gavini *et al.* 1989) into cotton. Here we conclusively demonstrated that the level of damage associated with SGSB feeding on greenhouse grown fruit depends on whether the insect harbours and transmits a bacterial pathogen of cotton. Ongoing efforts to further elucidate insect transmission of plant pathogens into cotton include (i) determining whether SGSB are mechanical or biological vectors of the pathogen, (ii) surgical dissection of stink bug tissue to identify pathogen reservoir(s) within the insects (i.e. feeding apparatus and/or digestive tract), (iii) determining whether transmission is favourable to specific bacterial pathogens; and (iv) assessing the level of damage to bolls of various maturities at the time of insect transmission of the plant pathogen.

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